Journal of Surgical Oncology 62:109–114 (1996)

Oxygenation in Tumors by Modified Hemoglobins

MUTSUMI NOZUE, MD, PhD, INTAE LEE, PhD, JAMES M. MANNING, PhD, LOIS R. MANNING, PhD, AND RAKESH K. JAIN, PhD

From Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts (M.N., R.K.J.); Department of Radiation Oncology, Cooper Hospital, University of Medicine & Dentistry of New Jersey, (I.L.), Camden, New Jersey; and the Laboratory of Biochemistry, Rockefeller University, (J.M.M., L.R.M.), New York, New York

The effect of systemic injection of modified hemoglobin (Hb) prepared from bovine, human, or mouse Hb on tumor oxygenation was investigated. Hb was modified by (1) diisothiocyanatobenzenesulfonate (DIBS) to yield cross-linking within a tetramer; (2) glycolaldehyde (Glyal) to yield cross-linking between and within tetramers; (3) carboxymethylation (Cm) to change oxygen affinity; or (4) poly(ethylene glycol) (PEG) to yield attachment between tetramers. HGL9 (human glioma) in nude mice and FSaII (mice fibrosarcoma) in C3H mice were used as tumor models. Dose and time dependency were detected in the oxygenation effect by bovine-PEG-Hb. Internal cross-linkage prolonged the half-life in the circulation, and thus showed a significant effect. Compared to bovine-CmHb, bovine-DIBS-Hb and bovine-DIBS-CmHb were more effective. Decreasing the oxygen affinity by Cm significantly enhanced tumor oxygenation. Human-DIBS-CmHb was more effective than human-DIBS-Hb. These effects were caused by oxygen carrying capacity of modified Hbs as well as hemodynamic factors, and the injection seemed to reduce both perfusion-limited (acute) and diffusion-limited (chronic) hypoxia.

KEY WORDS: **tumor oxygenation, modified hemoglobin, carbogen, Eppendorf "Histograph"**

INTRODUCTION

Oxygen tension in solid tumors has been reported to be low in human and rodent tumors [1]. Hypoxia in tumors may contribute to resistance to radiation and chemotherapy [2–4]. Hypoxic fraction in tumors has been related with the clinical outcome [5,6]. Several strategies have been attempted to increase oxygen tension in tumors, including the injection of modified hemoglobin (Hb) [7,8]. These studies reported that polymerized bovine Hb can increase the oxygen tension and enhance the radiation effect in rodent tumors. Since these studies only used one kind of modified Hb, the Hb parameters that can modify the oxygenation effects, such as molecular weight, half-life, and oxygen affinity, were not examined. Dose and time dependencies have not clearly been reported, either. Therefore, the purposes of this study were (1) to examine the dose and time dependencies of the oxygenation effect with modified Hb; (3) to evaluate the differences in oxygenation effect using several kinds of Hb that have different molecular weights, half-lives, and oxygen affinities; and (3) to measure the enhancement of the oxygenation effect of modified Hb by carbogen. This study may provide useful information on how and what kinds of modified Hb should be further investigated in clinical studies.

Abbreviations: Cm, carboxy-methylated; DIBS, diisothiocyanatobenzenesulfonate; Glyal, glycolaldehyde; Hb, hemoglobin; P_{50}, pressure of O_2 at which hemoglobin is 50% saturated; PEG, poly(ethylene glycol); TBF, tumor blood flow.

Accepted for publication February 4, 1996.

Address reprint requests to Dr. Mutsumi Nozue, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, 305 Japan.

TABLE I. Characteristics of Modified Hemoglobins and Effect of Injection on Arterial Blood Pressure and Gas

	Molecular weight	Half-life in circulation (hr)	P_{50} (mm Hg)	Blood pressure (mm Hg)	Arterial pO_2 (mm Hg)	Arterial pCO_2 (mm Hg)
Saline	—	—	—	95 ± 16	62 ± 25	49 ± 5
Mouse-CO-DIBS-Hb	64,000	n.d.[a]	n.d.	106 ± 16	n.d.	n.d.
Mouse-DIBS-Hb	64,000	n.d.	16	n.d.	n.d.	n.d.
Bovine-CmHb	64,000	n.d.[b]	n.d.	97 ± 17	61 ± 14	55 ± 8
Bovine-DIBS-CmHb	64,000	3	n.d.	125 ± 11*	60 ± 7	43 ± 6**
Bovine-Glyal-CmHb	>64,000	n.d.	n.d.	115 ± 11**	62 ± 6	49 ± 9
Bovine-PEG-Hb	>128,000	18[c]	18[d]	114 ± 13*	58 ± 13	53 ± 5
Human-DIBS-Hb[e]	64,000	3	9	n.d.	n.d.	n.d.
Human-DIBS-CmHb	64,000	n.d.	20	n.d.	n.d.	n.d.
Human-Glyal-CmHb	>64,000	n.d.	35	n.d.	n.d.	n.d.

[a]Not determined.
[b]Half-life of human-CmHb is 0.7 hr.
[c]Half-life in rat.
[d]p_{50} was obtained with one sample. Therefore, this is an approximate value.
[e]The DIBS-Hb used for the circulation half-life value was purified component B.
*$P < 0.01$, compared to the value after saline injection.
**$P < 0.05$, compared to the value after saline injection.

MATERIALS AND METHODS

Animals and Cell Lines

Animals included 8-week-old nude or C3H mice implanted with tumor chunks in the right leg. The experiments were done when the tumors grew to about 9 × 9 mm in size. HGL9 (human glioma) was implanted in nude mice and FSaII (mouse fibrosarcoma) in C3H mice. This study was approved by Massachusetts General Hospital Subcommittee on Research Animal Care.

Hb and Carbogen

Bovine, human, and mouse blood was used as a source of modified Hb. Native Hb is a tetramer with a molecular weight of 64,000. It has a short half life as discussed below. It also has a very high oxygen affinity. To modify these characteristics, several agents were used: to crosslink subunits within a tetramer, diisothiocyanatobenzenesulfonate (DIBS) was used [9,10]. This process increases the half-life from 0.7 to 3.3 hr. To crosslink or attach tetramers, glycolaldehyde (Glyal) [11] and poly(ethylene glycol) (PEG) [12] were used, respectively. Oxygen affinity was also changed by carboxymethylation (Cm) [11]. The concentration of Hb solution was from 6% to 8%. Molecular weight, oxygen affinity, i.e., pressure of O_2 at which hemoglobin is 50% saturated (P_{50}), and half-lives of some of modified Hb are summarized in Table I. Many data were not determined, but some of missing data would be speculated with reference to molecular weight and the method of modification. For example, stabilized Hbs with DIBS or Glyal would have almost the same half lives. Bovine-PEG-Hb was a kind gift of Enzon (Piscataway, NJ). Other Hb values were synthesized in the Laboratory of Biochemistry, Rockefeller University (New York, NY). To examine the effect of carbogen (mixture of 95% O_2 and 5% CO_2) breathing on oxygenation, mice were allowed to inhale carbogen for 5 min before and during pO_2 measurement.

pO_2 Measurement

The pO_2 measurements were done using a pO_2-Histograph (Eppendorf, Hamburg, Germany). Polarographic needle microelectrodes were calibrated in saline saturated with air (2 min) and 100% nitrogen (5 min). Modified Hb or saline was injected 1 hr before pO_2 measurements. Animals were anesthetized by ip injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) 30 min before pO_2 measurement. Room temperature, body temperature, respiration frequency and blood pressure (via canulation of carotid artery) were monitored. The animal was placed on a heating pad to maintain body temperature at 37–38°C. The reference electrode (Ag/AgCl ECG electrode) was attached to the abdominal skin. The skin and tumor capsule were perforated with a 23-gauge needle. The pO_2 microelectrode was positioned in the perforation and then allowed to move automatically 0.7 mm forward and 0.3 mm backward. Probe current was measured every 1.4 sec, and the probe moved forward again under computer control. After the probe reached the end of its measurement path, the probe was repositioned at the initial perforation site at a different angle and stepwise measurements were resumed. At least 40 points were monitored in each tumor. After the measurement, blood was obtained from the arterial cannula for blood-gas analysis (ABL330, Radiometer, Copenhagen, DK). Current between the needle type electrode and the anode were automatically converted to pO_2 values according to the calibration data.

Data Analysis

To analyze oxygen status in the tumor, the histogram of pO_2 distribution with a class width of 2.5 mm Hg was made for each tumor, and the cumulative fraction curve was drawn by summing up the columns successively from 0 mm Hg. From this curve, the fraction under a given oxygen tension, such as 2.5 mm Hg or 5 mm Hg, was obtained. The mean and standard error of these fractions from all tumors of one experimental group were calculated to obtain the cumulative curve of the group. To examine the difference, Student's *t*-test was applied to the mean of the fractions under given oxygen tension. Additionally, all pO_2 values obtained from several tumors of one cell line were grouped together and presented as a histogram.

RESULTS

Effect on Arterial Blood Pressure and Gas

Table I also summarizes the effect of mouse or bovine Hb injection on arterial blood pressure, pO_2 and pCO_2. Injection of stabilized Hb (DIBS, Glyal, or PEG Hb) significantly increased the blood pressure, but not arterial pO_2. Carbon monoxide-saturated mouse-DIBS-Hb also increased blood pressure, although not significantly. Bovine-CmHb injection did not change the blood pressure, presumably due to its short half life in circulation. Blood gas analysis did not show any systemic effect of Hb injection in comparison to controls, except for pCO_2 after bovine-DIBS-CmHb injection.

Dose Response

Figure 1A shows the cumulative fraction curves of oxygen distribution after the injection of 10 or 25 ml/kg of bovine-PEG-Hb in animals with HGL9 tumors. The hypoxic fraction decreased as the amount of injected Hb increased. The fraction under 2.5 mm Hg after the injection of 25 ml/kg of bovine-PEG-Hb was significantly lower than that after the injection of saline (Fig. 1B). Dose dependency was also observed in FSaII tumors, although differences among fractions under 2.5 mm Hg were not statistically significant (data not shown).

Time Dependence

As shown in Figure 2, pO_2 values in HGL9 tumors increased as a function of time after injection (25 ml/kg) of bovine-PEG-Hb. The median pO_2 value changed from 2.3 mm Hg (15 min) to 3.3 mm Hg (2 hr). The fractions under 2.5 mm Hg were 50 ± 32% (15 min) and 36 ± 34% (2 hrs), but this difference was not significant. The oxygen distribution after saline injection did not change with time. The same tendency was also measured followed injection of human-DIBS-CmHb and human-Glyal-CmHb; i.e., oxygen levels were higher at 1 hr compared to 15 min in HGL9 tumors [median pO_2 values were 2.4 (1 hr) vs. 1.6 (15 min) for human-DIBS-CmHb and 2.8 (1 hr) vs. 1.7 mm Hg (15 min) for human-Glyal-CmHb]. In FSaII tumors, oxygen status at 1 hr after injection of bovine-PEG-Hb was improved compared to the status at 2 or 3 hr after injection (median pO_2 values were 3.4, 1.8, and 2.2 mm Hg, respectively). The peak varied from 1 hr to 3 hr; no data were collected at more than 3 hr after injection.

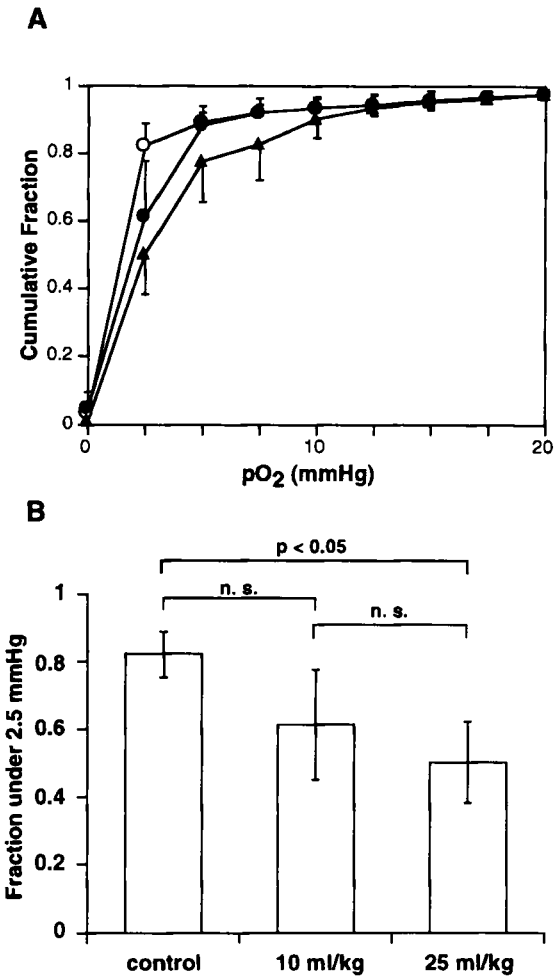

Fig. 1. Dose dependency of the oxygenation effect by bovine-PEG-Hb. HGL9 tumors were used for this study. **A:** Cumulative fraction curves obtained by 25 ml/kg (▲) or 10 ml/kg (●) of bovine-PEG-Hb, or saline (○) injection. Error bar: SE. **B:** Comparison of the fraction under 2.5 mm Hg in pO_2. There is a significant difference between the groups of 25 ml/kg of bovine-PEG-Hb and saline injection.

Effect of Modification of Hb on Tumor Oxygenation

Figure 3 shows the effect of Hb modification on tumor oxygenation. Cumulative curves obtained after the injection of modified bovine Hb are shown in Figure 3A. Compared to bovine-CmHb, bovine-DIBS-Hb and bovine-DIBS-CmHb were more effective. Internal cross-linkage showed most significant effect. In contrast, cross-

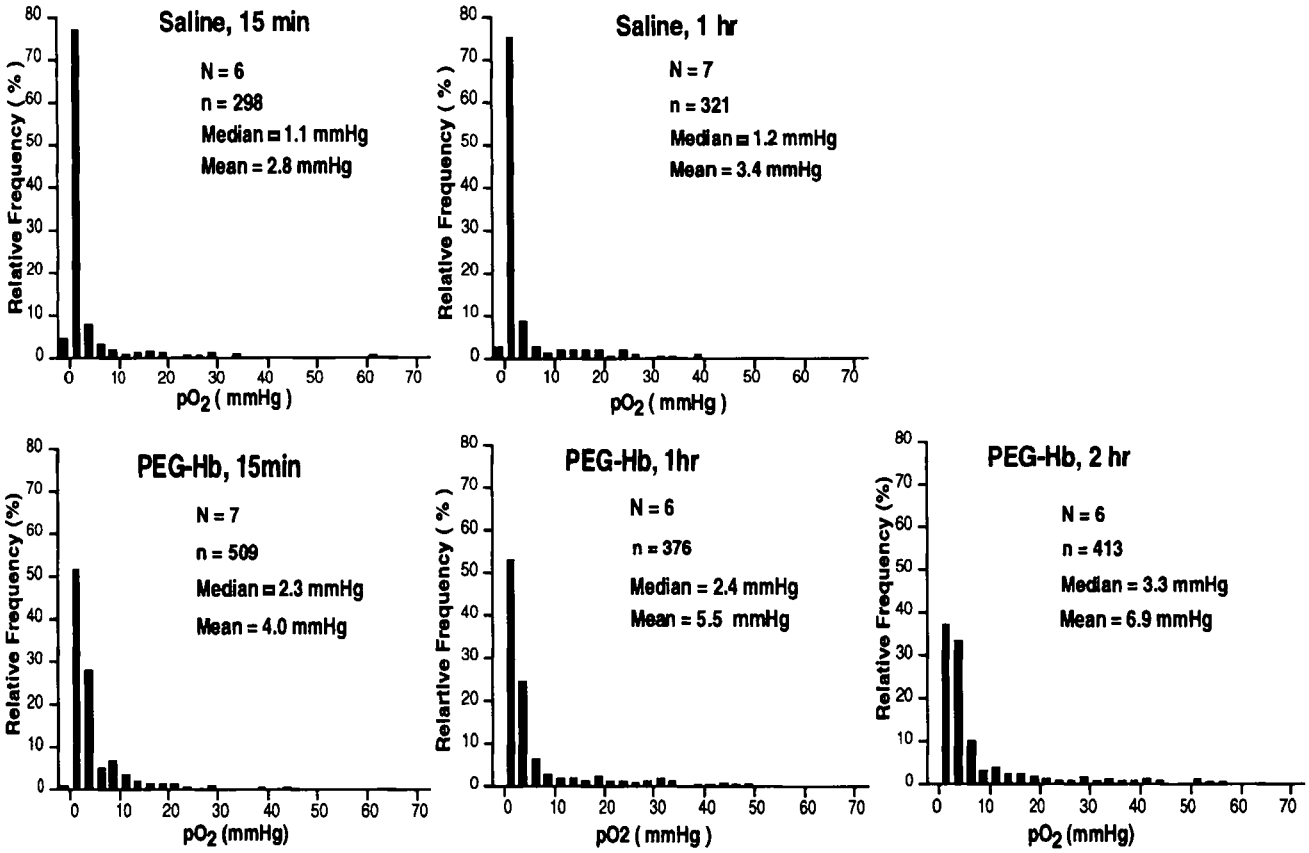

Fig. 2. Time course of oxygenation effect by bovine-PEG-Hb. Bovine-PEG-Hb or saline for control group (25 ml/kg) was injected to the mice with HGL9 tumors through the tail vein. pO_2 in tumors were measured 15 min, 1 or 2 hr after injection. About 50 pO_2 values were obtained from each tumor. All values in each experimental group were presented as a histogram with column widths of 2.5 mm Hg. N, the number of animals; n, the total number of pO_2 measurements; Median, the median value of pO_2 measurement; Mean, the mean value of pO_2 measurement.

linking of tetramers by Glyal reduced oxygenation. Bovine-PEG-Hb showed similar effect as bovine-DIBS-CmHb. In the case of human Hb, DIBS cross-linkage did not change oxygenation. Carboxymethylation, however, improved oxygenation (Fig. 3B). CO saturated mouse-DIBS-Hb did not oxygenate HGL9 tumors as mouse-DIBS-Hb did (data not shown).

Effect of Carbogen

As shown in Figure 4, carbogen significantly enhanced the oxygenation effects of bovine-PEG-Hb. The fractions under 2.5 mm Hg and 5 mm Hg were reduced by 30%, as compared to the control group ($P < 0.01$).

DISCUSSION

Red blood cell substitutes that can carry oxygen to the hypoxic tissues of an anemic body have been sought for some time [13]. First, perfluorochemicals, plasma-based oxygen carriers were tested for the feasibility in dogs with anemia and myocardial infarction [14]. In the area of radiation therapy, the oxygen status can significantly alter the outcome of radiation treatment in cancer patients. Thus, perfluorochemicals have been applied to increase oxygen tension in tumors. Lee et al. reported that the oxygen level in experimental tumors during the course of a single and/or fractionated irradiation significantly increased by an i.v. injection of perfluorochemical with carbogen inhalation [15,16]. Second, modified Hb and Hb-based oxygen carriers have been developed. As raw materials for Hbs, human and bovine Hbs have been routinely utilized. Pure unmodified Hb, however, tends to dissociate into dimers quite readily and its half life turns out to be less than 1 hr. This short retention time not only makes the material ineffective but also causes considerable kidney damage. Therefore, several agents have been used to change these characteristics [9,11,12], and encouraging results have been obtained. These modified Hbs have also been considered as oxygen status modifier in tumors [7].

Our results show that a single injection of various modified Hb can oxygenate tumors as reported [7]. The dose dependency obtained here further confirmed this

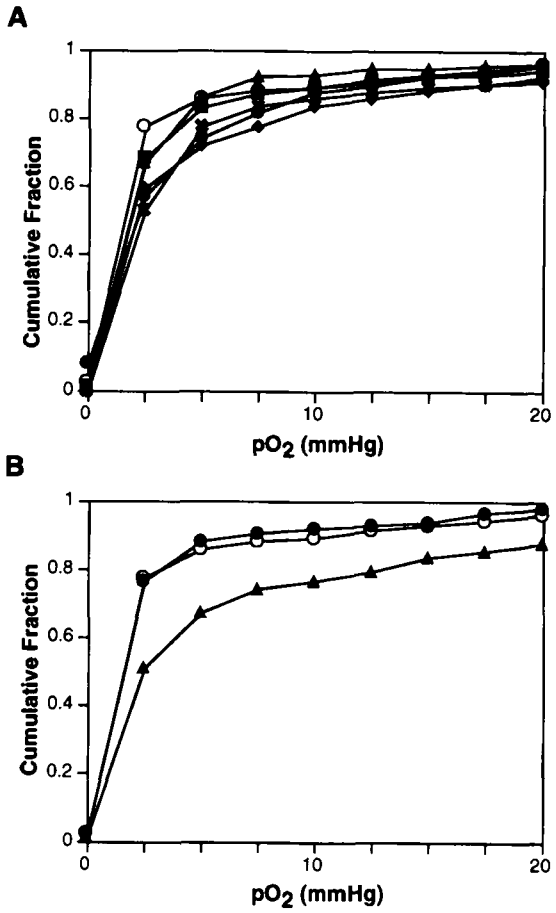

Fig. 3. Effect of modification of Hb on tumor (HGL9) oxygenation. PO_2 distribution of tumors treated by different kinds of Hb are presented as cumulative curves. The dose of Hb was 25 ml/kg, and pO_2 was measured 1 hr after injection. Error bar is not shown to avoid crowding. **A:** Effect of bovine Hbs on tumor oxygenation. saline (○), bovine-DIBS-Hb (●), bovine-CmHb (▲), bovine-DIBS-CmHb (◆), bovine-Glyal-CmHb (■), bovine-PEG-Hb (✖). **B:** Effect of human modified Hbs on tumor oxygenation. saline (○), human-DIBS-Hb (●), human-DIBS-CmHb (▲).

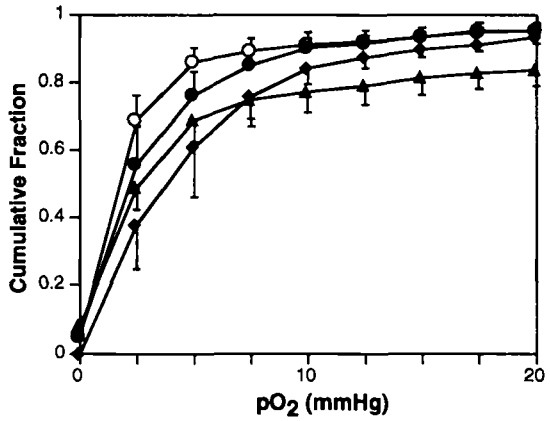

Fig. 4. Combined effect of bovine-PEG-Hb and carbogen on tumor (HGL9) oxygenation. Cumulative fraction curves were obtained by saline injection (○), bovine-PEG-Hb injection (●), carbogen inhalation for 5 min (▲), or both injection of bovine-PEG-Hb and inhalation of carbogen (◆). The dose of Hb was 25 ml/kg, and pO_2 was measured 1 hr after injection.

effect. The combined effect of carbogen and modified Hb was also similar. The appropriate interval between Hb injection and oxygenation was also estimated. Three different kinds of modified Hb showed better oxygenation effect at 1 hr after injection than at 15 min. In the HGL9 cell line, 2 hr interval showed the best effect, while in the FSaII 1 hr interval was the best. Thus, the timing of injection of modified Hb is important to achieve the maximal effect in tumor oxygenation.

What factors can influence the increase in oxygenation? Raised arterial blood pressure seemed to be one of the important factors, as indicated in Table I. The cause of raised blood pressure seems to be high oncotic pressure exerted by modified Hb solution, because the same increase in arterial pressure (120 mm Hg) was reported when 6% Dextran solution (hyperoncotic and iso-osmotic solution) was injected [17]. High arterial blood pressure can increase tumor blood flow (TBF) [18]. Thus, modified Hb values with longer half-lives showed better effect (bovine-CmHb vs. bovine-DIBS-CmHb in Fig. 3A). Second, injection of modified Hb can cause hemodilution. Hemodilution can reduce the viscosity, thus it can also increase TBF [13,18]. Hemodilution effect on oxygenation has been proved [17,19]. These two hemodynamic factors may affect so-called perfusion-limited (acute) hypoxia as a consequence of a vascular collapse which renders areas in tumors, that were well perfused only moment before, suddenly hypoxic [20].

In addition, the oxygen-carrying capacity of modified Hb is also suggested to be an important factor. For example, Cm can lead to better effect (human-DIBS-Hb vs. human-DIBS-CmHb in Fig. 3B), consistent with some studies showing that lowered oxygen affinity of Hb can oxygenate tumors [21,22]. Another example is that CO-mouse-DIBS-Hb did not oxygenate the tumor, while mouse-DIBS-Hb did. How does modified Hb improve oxygenation as a oxygen carrier? Possible explanations include the following:

1. Total oxygen carrying capacity of blood per unit should be increased. Since Hb concentration of modified Hb ranges from 6% to 8%, Hb concentration in whole blood would increase by 2–3 g/dl after the injection of Hb (25 ml/kg; about one-third of whole blood volume). Hb concentration 1 hr after injection should also be higher depending on the half life of injected modified Hb and hemoconcentration.
2. Hematocrit in capillary is lower than that in systemic circulation (Fahraeus effect). Thus, injected modified Hb can preferably increase Hb concentration in capillary than in systemic circulation. As an extreme

case, modified Hb can pass through RBC-free plasma channel in tumors, while red blood cells cannot. Additionally, raised arterial pressure may be able to open the collapsed vessels.

3. Modified Hb can extravasate from tumor vessels, especially in the peripheral part of the tumor, because the vessels in tumor are more permeable than normal capillaries [23,24]. Oxygen saturated Hb in interstitial space can release oxygen in hypoxic area. Therefore, the peripheral part of tumor may be better oxygenated than the central part.

These three mechanisms may be able to alleviate both perfusion-limited (acute) and diffusion-limited (chronic) hypoxia, in which large inter-capillary distances, resulting from rapid tumor cell proliferation, lead to hypoxic cells existing at the rim of the oxygen diffusion distance [20].

CONCLUSIONS

Injection of modified Hb oxygenated tumors. This effect depended on dose and type of modification of Hb. It can oxygenate both diffusion and perfusion-limited regions through hemodynamic effect and increased oxygen carrying capacity. The detailed mechanism of oxygenation needs to be investigated using microcirculation techniques.

ACKNOWLEDGMENTS

We thank Ms. Sylvie Roberge and Ms. Julia Kahn for their excellent technical assistance. We also thank Drs. Herman D. Suit, Yves Boucher, Gabriel Helmlinger, and Marc Dellian for their helpful comments. This work was supported by grants R35-CA-56591 and CA-13311 from NIH and by a gift from Enzon, Inc.

REFERENCES

1. Kallinowski F, Zander R, Höckel M, Vaupel P: Tumor tissue oxygenation as evaluated by computerized-pO_2-histography. Int J Radiat Oncol Biol Phys 19:952–961, 1990.
2. Kennedy K, Teicher B, Rockwell S, Sartorelli A: The hypoxic tumor cell: A target for selective cancer chemotherapy. Biochem Pharmacol 29:1–8, 1980.
3. Teicher BA, Rose CM: Effect of dose and scheduling on growth delay of the Lewis lung carcinoma produced by the perfluorochemical emulsion, Fluosol-DA. Int J Radiat Oncol Biol Phys 12:1311–1313, 1986.
4. Teicher BA, Lazo JS, Merrill WW, et al.: Effect of Fluosol-DA/O_2 on the antitumor activity and pulmonary toxicity of bleomycin. Cancer Chemother Pharmacol 18:213–218, 1986.
5. Gatenby R, Kessler H, Rosenblum J, et al.: Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. Int J Radiat Oncol Biol Phys 14:831–838, 1988.
6. Höckel M, Knoop C, Schlenger K, et al.: Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol 26:45–50, 1993.
7. Teicher BA, Schwartz GN, Alvarez-Sotomayor E, et al.: Oxygenation of tumors by a hemoglobin solution. J Cancer Res Clin Oncol 120:85–90, 1993.
8. Teicher B, Herman T, Hopkins R, Menon K: Effect of oxygen level on the enhancement of tumor response to radiation by perfluorochemical emulsions or a bovine hemoglobin preparation. Int J Radiat Oncol Biol Phys 21:969–974, 1991.
9. Manning LR, Morgan S, Beavis RC, et al.: Preparation, properties, and plasma retention of human hemoglobin derivatives: Comparison of uncrosslinked carboxymethylated hemoglobin with crosslinked tetrameric hemoglobin. Proc Natl Acad Sci USA 88:3329–3333, 1991.
10. Manning JM: Design of chemically modified and recombinant hemoglobins as potential red cell substitutes. In Winslow RM, Vandegriff KD, Intaglietta M (eds): "Blood Substitutes: Physiological Basis of Efficacy." Boston: Birkhäuser, 1995, p 76–89.
11. Manning LR, Manning JM: Influence of ligation state and concentration of hemoglobin A on its cross-linking by glycoaldehyde: Functional properties of cross-linked, carboxymethylated hemoglobin. Biochemistry 27:6640–6644, 1988.
12. Nho K, Zalipsky S, Abuchowski A, Davis FF: PEG-modified hemoglobin as an oxygen carrier. In Harris JM (eds): "Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications." New York: Plenum Press, 1992, p 171–182.
13. Tsai AG, Kerger H, Intaglietta M: Microcirculatory consequences of blood substitution with aa-hemoglobin. In Winslow RM, Vandegriff KD and Intaglietta M (eds): "Blood Substitutes: Physiological Basis of Efficacy." Boston: Birkhäuser, 1995, p 155–174.
14. Glogar D, Kloner R, Muller J, et al.: Fluorocarbons reduce myocardial ischemic damage after coronary occlusion. Science 211:1439–1441, 1981.
15. Lee I, Levitt S, Song C: Radiosensitization of murine tumors by Fluosol-DA 20%. Radiat Res 122:275–279, 1990.
16. Lee I, Cunningham W, Levitt S: Improvement in RBC flux, acidosis and oxygenation in tumour microregions by Fluosol-DA 20%. Int J Radiat Biol 60:695–705, 1991.
17. Lee I, Demhartner TJ, Boucher Y, et al.: Effect of hemodilution and resuscitation on tumor interstitial fluid pressure, blood flow, and oxygenation. Microvasc Res 48:1–12, 1994.
18. Jain RK: Determinants of tumor blood flow: A review. Cancer Res 48:2641–2658, 1988.
19. Jung C, Müller-Klieser W, Vaupel P: Tumor blood flow and O_2 availability during hemodilution. Adv Exp Med Biol 180:281–291, 1984.
20. Siemann DW: Keynote address: Tissue oxygen manipulation and tumor blood flow. Int J Radiat Oncol Biol Phys 22:393–395, 1992.
21. Siemann D, Macler L: Tumor radiosensitization through reductions in hemoglobin affinity. Int J Radiat Oncol Biol Phys 12:1295–1297, 1986.
22. Hirst DG, Wood PJ: The influence of haemoglobin affinity for oxygen on tumour radiosensitivity. Br J Cancer 55:487–491, 1987.
23. Yuan F, Leunig M, Berk DA, Jain RK: Microvascular permeability of albumin, vascular surface area, and vascular volume measured in human adenocarcinoma LS174T Using dorsal chamber in SCID mice. Microvasc Res 45:269–289, 1993.
24. Dvorak H, Nagy J, Dvorak J, Dvorak A: Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am J Pathol 133:95–109, 1988.